Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 852 (2007) 202–207
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Liquid chromatography–tandem mass spectrometry assay for the quantitation of β-dihydroartemisinin in rat plasma

Jie Xing [a], Hong-Xia Yan [b], Rui-Li Wang [b], Li-Feng Zhang [b], Shu-Qiu Zhang [b,*]

[a] *School of Pharmaceutical Sciences, Shandong University, Jinan 250012, China*
[b] *School of Pharmaceutical Sciences, Shanxi Medical University, Taiyuan 030001, China*

Received 1 October 2006; accepted 10 January 2007
Available online 18 January 2007

Abstract

Dihydroartemisinin (DHA) is a sesquiterpene used in the world as an antimalarial. To evaluate the pharmacokinetics of dihydroartemisinin in rats, a sensitive and specific liquid chromatography/tandem mass spectrometric (LC–MS/MS) method was developed and validated for the quantitation of dihydroartemisinin in rat plasma. For detection, a Sciex API 4000 LC–MS/MS with a TurboIonSpray ionization (ESI) inlet in the positive ion-multiple reaction monitoring (MRM) mode was used. The plasma samples were pre-treated by a simple liquid–liquid extraction with diethyl ether. The statistical evaluation for this method reveals excellent linearity, accuracy and precision for the range of concentrations 0.2–100.0 ng/mL. The method had a lower limit of quantification (LLOQ) of 0.2 ng/mL for β-dihydroartemisinin in 100 μL of plasma. The method was successfully applied to the characterization of the pharmacokinetic profile of β-dihydroartemisinin in rats after oral administration.

Keywords: Dihydroartemisinin; Liquid chromatography–tandem mass spectrometry; Pharmacokinetics; Rat plasma

1. Introduction

Dihydroartemisinin (DHA, Fig. 1) is the lactol reduction product of artemisinin (qinghaosu), which has potent antimalarial activity *in vitro* and *in vivo* [1,2]. It has been widely reported that semi-synthetic derivatives of artemisinin can be metabolized in liver to its major metabolite DHA, which possesses greater antimalarial potency than the parent drug [3]. Therefore, it is important to monitor DHA in addition to parent drug for pharmacokinetic studies of artemisinin derivatives, such as artemether, arteether and artesunate.

Several analytical techniques have been reported for qualitative and quantitative determination of dihydroartemisinin and its analogues in biological matrices [4–6]. However, long run duration, complicated sample preparation and inadequate sensitivity characterize the previous methods. Liquid chromatography coupled with mass spectrometry (LC–MS), using different modes of ionization, has been described in literature for the quantitation of DHA and its analogues [2,7–9]. These LC–MS methods dealt with simultaneous estimation of artemisinin derivatives, such as artemether, arteether or artesunate, and its metabolite DHA. However, the analytes were mostly quantified in selected ion monitoring mode (SIM), which provides relatively lower sensitivity than multiple reaction monitoring mode (MRM). A reported sensitive LC–MS assay for the simultaneous quantification of arteether and DHA in plasma was conducted in MRM mode, which gave a lower limit of quantification (LLOQ) of 0.78 ng/mL [7].

The pharmacokinetics and bioavailability of DHA in rats were studied previously using HPLC with reductive electrochemical detector, and the LLOQ of DHA was 10 ng/mL [10]. In the present work, a more sensitive and simple method with shorter analysis time (4 min/per sample) using LC–MS/MS was developed for quantitation of β-DHA in rat plasma. The pharmacokinetic profile of β-DHA in rats after an oral administration of β-DHA 10 mg/kg was investigated.

* Corresponding author at: School of Pharmacy, Shanxi Medical University, 56# Xinjian South Road, Taiyuan 030001, China. Tel.: +86 351 4690140; fax: +86 351 4690140.
E-mail addresses: shuqiu.zhang@sohu.com, shuqiu.zhang@sxmu.edu.cn (S.-Q. Zhang).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.01.017

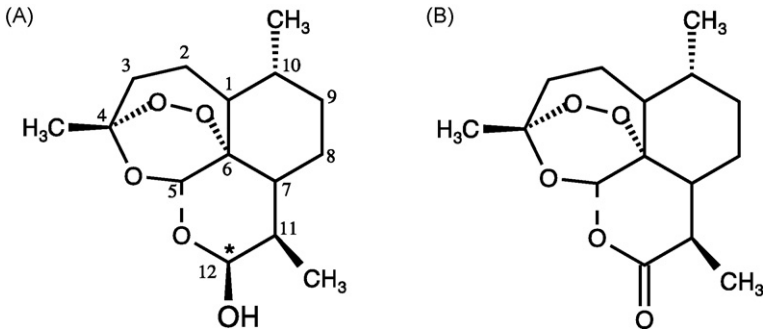

Fig. 1. Chemical structures of β-dihydroartemisinin (A) and artemisinin (internal standard, B).

2. Experimental

2.1. Chemicals and reagents

Dihydroartemisinin and artemisinin (as internal standard, IS, Fig. 1) were provided by Kunming Pharmaceutical Co. (purity >99.0%, Yunnan, China). Based on the ^{1}H NMR, ^{13}C NMR and LC–MS/MS data, DHA reference is found to be a pure β-epimer. Acetonitrile (HPLC grade) was purchased from Fisher Chemicals (Fairlawn, NJ, USA). Diethyl ether was of analytical grade from Tianda Chemicals Ltd. (Tianjin, China). Other chemicals used were of analytical reagent grade and purchased from commercial sources. Water was deionized, filtered and purified (Resistivity >18 MΩ cm) on a Milli-Q Reagent Grade Water System (Millipore Corporation, Bedford, MA, USA).

2.2. Instrumentation

The HPLC was performed using an Agilent 1100 system (PaloAlto, CA, USA) equipped with a G1313A auto-sampler, a vacuum degasser unit and a G1312A binary pump. The HPLC system was coupled to an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a TurboIonSpray ionization (ESI) interface for mass analysis and detection. A 10-port switching valve (Rheodyne, Cotati, CA, USA) was used to direct HPLC eluate to waste in the first 2.5 min of the chromatographic run, and afterwards to the ionization source. Data were collected and analyzed by the Analyst 1.3 data acquisition and processing software (Applied Biosystems/MDS Sciex).

NMR spectroscopic data of DHA reference were obtained in d6-DMSO. ^{1}H and ^{13}C spectra were obtained with a Bruker Avance 600 spectrometer operating at 600 and 150 MHz, respectively.

2.3. Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved on a Luna ODS C_{18} column (150 mm × 4.6 mm i.d., 5 μm; Phenomenex, Torrance, CA, USA) with a 4.0 mm × 3.0 mm i.d. SecurityGuard C_{18} (5 μm) guard column (Phenomenex, Torrance, CA, USA). The chromatography was performed at 20 °C. The mobile phase consisted of acetonitrile/10 mM aqueous ammonium acetate containing 0.1% (v/v) formic acid (85:15, v/v), delivered at a flow rate of 0.8 mL/min.

The mass spectrometer was operated in positive ion mode. The tuning parameters were optimized for β-DHA and artemisinin (IS) by infusing an acetonitrile solution containing 100 ng/mL of analytes at a flow rate of 30 μL/min into the mobile phase (0.8 mL/min) using a T connection. Following optimization of the settings, the instrument was operated with an ion spray voltage of +5.5 kV, backpressures for collision gas of 3 psi, curtain gas of 12 psi, nebulizer gas of 50 psi and heater gas of 50 psi; the heater gas temperature was set at 550 °C. Ultrapure nitrogen was used as nebulizer, heater, curtain and collision-activated dissociation (CAD) gas. The fragmentation transitions for the multiple reaction monitoring were *m/z* 267.4–163.4 for β-DHA, and *m/z* 300.4–209.4 for the IS, with a dwell time of 200 ms per transition.

2.4. Preparation of calibration standards and quality control samples

Stock solutions of β-DHA and IS were prepared by dissolving each of the accurately weighed reference compound in acetonitrile. The primary stock solution of β-DHA (400 μg/mL) was prepared in acetonitrile and diluted with acetonitrile to give working solutions of 0.2, 0.5, 2, 5, 20, 50 and 100 ng/mL. Three quality control-method validation stock solutions were prepared from a separate weighing of β-DHA. Dilutions were used to prepare three levels of QC working solutions, 0.5, 5 and 80 ng/mL. The IS (artemisinin) solution of 200 ng/mL was similarly prepared by diluting the stock solutions of IS (100 μg/mL) with acetonitrile. All stock solutions and working solutions were stored at 4 °C.

Calibration standards and quality control samples in the concentration range of 0.2–100 ng/mL were prepared for calibration, accuracy and precision, quality control and stability assessment. Calibration standards and QC samples were prepared by spiking 100 μL of working solutions and 20 μL of IS (200 ng/mL) with 100 μL of drug-free plasma and 200 μL of phosphate buffer (pH 7.4). This mixture was extracted with 3 mL of diethyl ether by shaking for 10 min. The upper organic and lower aqueous phases were separated by centrifugation at 3000 *g* for 10 min. The organic phase was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was dissolved in 100 μL of the mobile phase

and vortex-mixed for 1 min. A 10-μL aliquot of the solution was injected into the LC–MS/MS system for analysis. Matrix matched calibration standards were obtained with concentrations of 0.2, 0.5, 2, 5, 20, 50 and 100 ng/mL of β-DHA in plasma. QC samples were obtained with three concentration levels of 0.5, 5 and 80 ng/mL of β-DHA in plasma.

2.5. Sample preparation

To 100 μL of rat plasma, 20 μL of the IS (200 ng/mL) and 100 μL of acetonitrile were added, and then 200 μL of phosphate buffer (pH 7.4) was added and mixed. This mixture was extracted with 3 mL of diethyl ether and treated as above. Plasma samples were diluted with blank plasma and re-analyzed when the concentration of β-DHA was higher than the upper limit of quantification (ULOQ) (100 ng/mL).

2.6. Application to pharmacokinetic study

Male Wistar rats (230–250 g) were supplied by Lab Animal Center of Shandong University (Grade II, Certificate No. SYXK 2003–0004). Rats were maintained at 22 ± 2 °C and 55 ± 5% relative humidity on a 12-h light:12-h dark cycle for at least 5 days before being used. Rats were fasted for 12 h before drug administration and for a further 3 h after dosing. Water was freely available for rats during experiments. β-DHA dissolved in sesame-oil was given orally to rats ($n = 6$, 10 mg/kg). After oral administration, blood samples of 250 μL were withdrawn from one jugular vein before dosing and at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 4.0 and 5.0 h post-dosing. After each sampling, 250 μL of physiologic saline was replenished to the blood from another jugular vein. All heparinized blood samples were centrifuged at 3000 *g* for 15 min, and plasma was obtained. All the plasma samples were stored at −20 °C and assayed within 1 month.

The LC–MS/MS assay developed was used to investigate the plasma concentration–time profile of β-DHA after an oral dose of β-DHA 10 mg/kg in rats.

2.7. Data analysis

The peak plasma concentration (C_{max}) and the time to peak concentration (t_{max}) were obtained from experimental observations. The other pharmacokinetic parameters were analyzed by a non-compartmental model with the aid of the TOPFIT program (Version 2.0, Thomae GmbH, Germany). The area under the plasma concentration–time curve (AUC_{0-t}) was calculated using the linear trapezoidal rule to the last point. The mean residence time (MRT) was obtained by dividing the area under the first moment–time curve ($AUMC_{inf}$) by the area under the curve (AUC_{inf}). Oral total body clearance (CL/F) was calculated as dose/AUC_{inf}.

3. Results and discussion

3.1. Method development

The influence of the mobile phase modifier and pH on the signal intensity was assessed. Based on the results, the mobile phase composed of 10 mM ammonium acetate buffer at pH 4 and acetonitrile was selected. Because DHA analogues such as artemether, arteether and artesunate can be converted to DHA *in vivo* [7–10], it is not suitable to use them as IS of DHA. Considering the similarity of physical–chemical properties and impossibility of interconversion *in vivo*, artemisinin was selected as IS in this experiment.

When β-DHA and IS were injected directly into the mass spectrometer along with the mobile phase in the positive mode, protonated molecules $[M+H]^+$ of β-DHA and IS were not observed in abundance, but the fragment of the β-DHA by loss of a H_2O and the adduct ion $[M+NH_4]^+$ of IS were observed. Under these conditions, the analytes yielded predominantly $[M+H-H_2O]^+$ ion at *m/z* 267.4 for β-DHA and ammonium adduct ion $[M+NH_4]^+$ at *m/z* 300.4 for IS, the internal standard. Each of the precursor ions was subjected to collision-induced dissociation to determine the resulting product ions. Interface independent instrument parameters were optimized during the infusion of a solution of β-DHA and IS through the TurboIonSpray (TISP) interface with HPLC mobile phase. The product ion spectra for β-DHA and IS are shown in Fig. 2.

The sample clean-up involved addition of acetonitrile, phosphate buffer and extraction organic solvent. The processes of protein precipitation and extraction gave a high and stable recovery of β-DHA and IS.

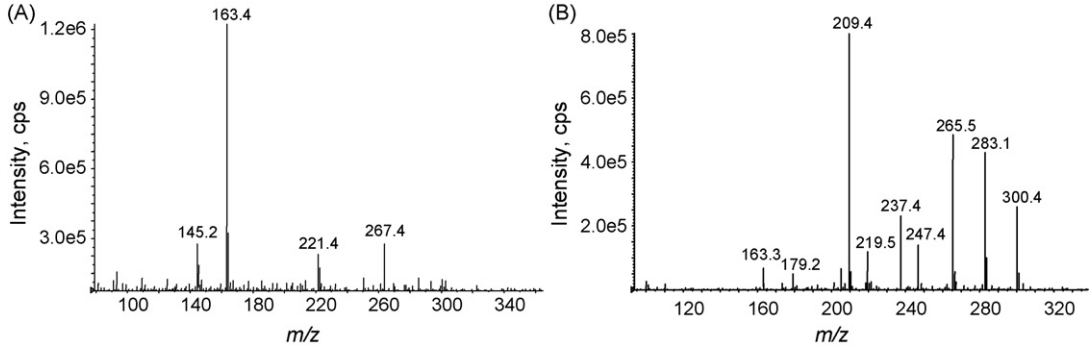

Fig. 2. Product ion mass spectra of the $[M+H-H_2O]^+$ ions of β-dihydroartemisinin (A) and the $[M+NH_4]^+$ ions of artemisinin, the internal standard (B).

3.2. Separation and relative retention time

The proton-NMR spectra data of DHA reference showed the characteristic shifts of 12- and 5-protons at 4.97 ppm (1H, t, $J = 3.6$, 12-H) and 5.43 ppm (1H, s, 15-H), respectively. The coupling between H-12 and H-11 has been used previously to assign the stereochemistry of the configuration of DHA and its derivatives [11,12]. For DHA, the coupling constant would be about 3.6 Hz if the hydroxyl substituent was in the β position and 9.2 Hz when the hydroxyl group was in the α position. Obviously, the 12-proton of DHA reference appeared as a simple triplet with a small coupling constant. Thus, it would be a pure β-epimer. The ^{13}C NMR spectrum revealed that DHA reference contained 15 carbons with chemical shifts similar to β-epimer of DHA.

Under optimized HPLC and MS conditions, β-dihydroartemisinin and artemisinin (IS) were detected at the retention times of 3.16 and 3.50 min, respectively (Fig. 3). Since no late-eluting peaks were observed, regeneration of the column using a gradient elution step was not necessary. The total run time was 4.0 min. In a previous study, both α- and β-epimer were measured in plasma, and it was found there was no significant difference in the peak area ratio of α-DHA to β-DHA between plasma spiked samples (3.25) and patient plasma samples (3.30). Considering that DHA administrated in present experiment is β-epimer, main attention was paid to β-DHA. Only β-epimer was quantified, and α-DHA was not measured under present conditions.

Blank rat plasma from six lots showed no significant interfering peaks at the retention times of β-DHA and IS. Since plasma samples were precipitated with acetonitrile and then extracted with diethyl ether, the background was very low and reconstituted samples did not cause any type of tubing or needle sprayer clog caused by original proteins. Matrix effects from co-eluting endogenous substances provide another possible source of problems regarding assay specificity, although

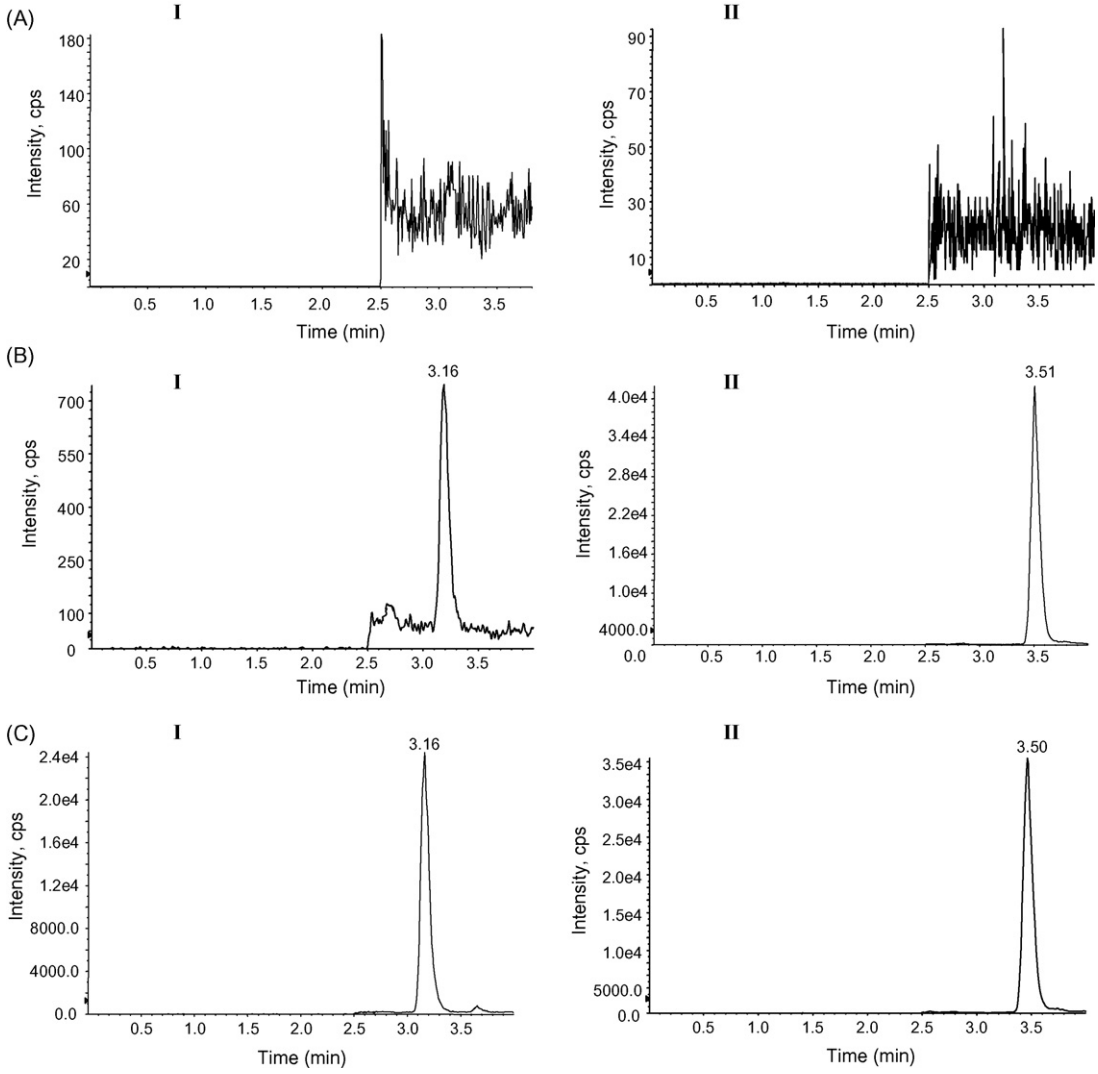

Fig. 3. Representative MRM chromatograms of: (A) a blank rat plasma sample; (B) a blank rat plasma sample spiked with β-dihydroartemisinin (0.2 ng/mL) and IS (artemisinin, 200.0 ng/mL); (C) a rat plasma sample at 0.75 h after an oral dose of β-dihydroartemisinin (10 mg/kg) to rats. I: β-dihydroartemisinin (*m/z* 267.4 → 163.4); II: IS (*m/z* 300.4 → 209.4).

matrix-matched calibration standards were used. The ion suppression effect was evaluated by comparing the peak areas of β-DHA (six QC samples of three concentration levels) and the IS (200 ng/mL) obtained from blank plasma extracts spiked with reference solutions with that of reference solutions at same concentration levels in acetonitrile, which was used as the reconstitution solution. For β-DHA, the mean peak areas from the six QC samples had relative error of 4.2%, when compared with that of QC working solutions. For the IS, the relative error was −3.4%. These observations indicate that endogenous substances did not significantly influence the ionization of β-DHA and IS.

Carryover effect was tested by injecting the high concentration standard (100 ng/mL for β-DHA and IS) in system followed by acetonitrile injection. No analytes were detected in the acetonitrile injection, which indicated the absence of carryover effect.

3.3. Linearity

The calibration curve was linear over the concentration range of 0.2–100.0 ng/mL of β-DHA in rat plasma with correlation coefficients $r > 0.99$ and consistent slope values when evaluated by weighed ($1/x^2$) least squares linear regression. Residuals were randomly distributed when plotted against concentration. A typical equation of the calibration curve was as follows: $y = 5.030 \times 10^{-3} + 9.560 \times 10^{-3}\ x$, $r = 0.9994$, where y represents the peak area ratios of β-DHA to that of IS, and x represents the plasma concentrations of β-DHA. The lower limit of quantification of β-DHA in rat plasma was established at 0.2 ng/mL. The LLOQ was accepted as the lowest points on the standard curve with a relative standard deviation of less than 15% and signal to noise ratio of 5:1 for the analysis for β-DHA. Results at the lowest concentration studies met the criteria for limit of quantitation. A typical chromatogram of an LLOQ sample is shown in Fig. 3.

3.4. Accuracy and precision

The accuracy and precision of the method were assessed by determining lower limit of quantification (0.2 ng/mL), QC (0.5, 5 and 80 ng/mL) and upper limit of quantification (100 ng/mL) samples using six replicated preparations of plasma samples at five concentration levels, respectively. Intra-day accuracy and precision were evaluated on the same day. To assess the inter-day accuracy and precision, the intra-day assays were repeated on three different days. Table 1 shows the results of calibration accuracy and the intra-day and inter-day precision in the 3-day validation study. The method was found to be highly accurate with deviation <6.4% from the nominal values and highly precise with intra-day precision <6.2% and inter-day precision <5.7% at each concentration of QC, LLOQ and ULOQ samples tested.

Table 1
Intra-day and inter-day precision and accuracy for β-dihydroartemisinin in rat plasma ($n = 3$ days, six replicates per day)

Added concentration (ng/mL)	Accuracy (%CV)		Precision (%CV)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.2	4.8	5.1	4.5	5.6
0.5	1.9	2.3	3.2	3.9
5	4.2	3.6	6.2	5.7
80	5.6	6.4	2.4	4.5
100	2.4	3.2	3.9	2.6

3.5. Recovery

The extraction recoveries of β-DHA and IS were determined at three QC levels by comparing the peak area of each analyte in plasma samples that had been spiked with the analytes prior to extraction with those for samples to which the analytes had been added post-extraction. The mean recovery after liquid–liquid extraction with diethyl ether was 92.3 ± 5.6% and 95.4 ± 4.5% for β-DHA and IS, respectively. These results suggested that there was no relevant difference in extraction recovery at different concentration levels for both β-DHA and IS.

3.6. Stability

Stability was assessed by leaving the QC samples of three different concentrations under several conditions, and all the QC samples for stability assessment were analyzed in triplicate. There was no significant difference (%CV <15%) between the responses of standards at time zero and after storage of plasma at room temperature on the bench-top for at least 4 h in terms of %CV (5.6%) for β-DHA. Processed samples were stable up to 24 h in the auto-sampler tray with the %CV value of 7.3%. Plasma samples were stable at −20 °C for at least 4 weeks with no significant loss (<4.8%). Plasma samples were stable over at least three freeze/thaw cycles in terms of %CV (5.9%). The stability data are shown in Table 2.

3.7. Application to rat plasma sample analysis

The method was successfully applied to the quantitation of β-DHA in rat plasma after an oral administration of β-DHA 10 mg/kg. The mean (+S.D.) ($n = 6$) plasma concentration versus time profile for β-DHA is depicted in Fig. 4. The mean values (±S.D.) of C_{max}, t_{max}, AUC_{0-t}, MRT and CL/F of β-

Table 2
Stability data of β-dihydroartemisinin in plasma or solutions

Concentration (ng/mL)	Bench-top (4 h, %CV)	Auto-sampler (24 h, %CV)	Long-term (−20 °C, 1 month, %CV)	Three freeze–thaw (%CV)
0.5	5.6 ± 1.8	6.1 ± 1.9	4.8 ± 1.5	5.9 ± 1.2
5	4.8 ± 1.6	7.3 ± 2.5	4.2 ± 2.1	4.3 ± 2.2
80	5.2 ± 2.1	5.6 ± 2.4	3.6 ± 1.8	4.8 ± 1.7

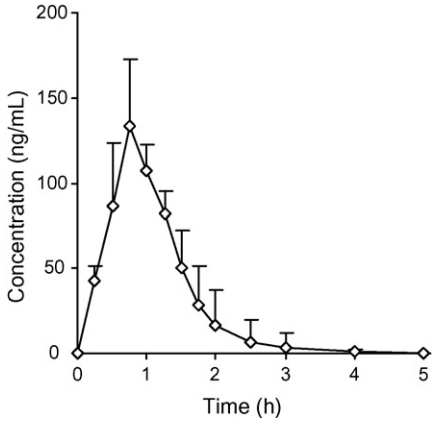

Fig. 4. Mean (+S.D.) plasma concentration–time profile of β-dihydroartemisinin in rats ($n = 6$) following an oral dose of 10 mg/kg of β-dihydroartemisinin.

DHA were 142.2 ng/mL (±21.1), 0.8 h (±0.1), 145.8 ng h/mL (±33.6), 1.0 h (±0.2) and 1.19 L/min/kg (±0.25), respectively.

4. Conclusions

The present optimized LC–MS/MS method was validated to guarantee a reliable quantitation of β-dihydroartemisinin in rat plasma after an oral dose of 10 mg/kg. The assay procedure is simple and relatively short allowing sufficient sample throughput to be applied to pharmacokinetic studies of β-dihydroartemisinin. The results of validation show that the method is reproducible and accurate. The analysis requires only 0.1 mL of plasma, which is an advantage in pharmacokinetic studies. This LC–MS/MS method has an LLOQ of 0.2 ng/mL for β-dihydroartemisinin and the analysis time is 4 min per sample, which are superior to previous analytical methodologies.

Acknowledgement

This work was supported by Grant 30572367 of the National Natural Science Foundation of China.

References

[1] K.F. Ilett, B.T. Ethell, J.L. Maggs, T.M.E. Davis, K.T. Batty, B. Burchell, T.Q. Binh, L.T.A. Thu, N.C. Hung, M. Pirmohamed, B.K. Park, G. Edwards, Drug Metab. Dispos. 30 (2002) 1005.
[2] H. Naik, D.J. Murry, L.E. Kirsch, L. Fleckenstein, J. Chromatogr. B 816 (2005) 233.
[3] J.M. Grace, A.J. Aguilar, K.M. Trotman, T.G. Brewer, Drug Metab. Dispos. 26 (1998) 313.
[4] J. Karbwang, K. Na-Bangchang, P. Molunto, V. Banmairuroi, K. Congpuong, J. Chromatogr. B 690 (1997) 259.
[5] D.K. Muhia, E.K. Mberu, W.M. Watkins, J. Chromatogr. B 660 (1994) 196.
[6] K.T. Batty, T.M.E. Davis, L.T.A. Thu, T.Q. Binh, T.K. Anh, K.F. Ilett, J. Chromatogr. B 677 (1996) 345.
[7] S. Sabarinath, M. Rajanikanth, K.P. Madhusudanan, R.C. Gupta, J. Mass Spectrom. 38 (2003) 732.
[8] C. Souppart, N. Gauducheau, N. Sandrenan, F. Richard, J. Chromatogr. B 774 (2002) 195.
[9] M. Rajanikanth, K.P. Madhusudanan, R.C. Gupta, Biomed. Chromatogr. 17 (2003) 440.
[10] Q.G. Li, J.O. Peggins, L.L. Fleckenstein, K. Masonic, M.H. Heiffer, T.G. Brewer, J. Pharm. Pharmacol. 50 (1998) 173.
[11] J.K. Baker, J.D. McChesney, H.T. Chi, Pharm. Res. 10 (1993) 662.
[12] R.K. Haynes, H.W. Chan, M.K. Cheung, W.L. Lam, M.K. Soo, H.W. Tsang, A. Voerste, I.D. Williams, Eur. J. Org. Chem. 1 (2002) 113.